Microb. Resour. Syst. **37**(1): 13-20, 2021

Long-term cryopreservation of basidiomycete strains with Homolka's perlite protocol followed by storage in an ultra-low-temperature freezer and vapor-phase liquid nitrogen tank

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We evaluated the utility of Homolka's perlite protocol (HPP) for the long-term cryopreservation of 105 strains of 33 ectomycorrhizal basidiomycete species maintained at the National Institute of Technology and Evaluation's Biological Resource Center in an ultra-low-temperature (−80°C) freezer. Of the 105 strains, 86 were viable and 19 revived poorly after 5 years storage at −80°C. The effects of the storage temperature on viability and hyphal growth after freezing-thawing after 5 or 6 years of preservation were evaluated in 25 strains that showed poor or fluctuating viability. Of these, seven strains had better revival rates after storage at −170°C than at −80°C, and the colony diameters of the revived cultures were significantly larger after storage at −170°C than after storage at −80°C. Thus, HPP is a useful long-term preservation method for many ectomycorrhizal basidiomycete cultures, and a lower storage temperature (−170°C) resulted in better survival and viability of the frozen cultures.

Key words: basidiomycete, cryopreservation, long-term preservation, perlite

INTRODUCTION

Mushrooms are important forestry and agricultural products worldwide. The mycorrhizal basidiomycetes that form edible mushrooms are important biological resources, although many species have not been cultivated successfully. To maintain these cultures, various cryopreservation methods have been examined and some have been used successfully in many laboratories and culture collections (Homolka, 2014). Despite these efforts to improve cryopreservation methods, many cryosensitive cultures still cannot be revived from the frozen state (Ito & Nakagiri, 1996; Homolka, 2014).

The National Institute of Technology and Evaluation's Patent Microorganism Depositary usually accepts microorganism deposits as frozen or dried cultures (freeze-dried or L-dried ampoules), but will accept active cultures of difficult-to-preserve strains and maintain them by subculturing, which is costly, increases the risk of contamination, and carries a high risk of genetic drift during storage (Smith, 1991; Ryan & Smith, 2004; Sakurai, *et al*., 2019). Thus, the development of a reliable long-term cryopreservation method is required to preserve such difficult-to-preserve strains. Recently, we improved Homolka's perlite protocol (HPP) (Homolka *et al*., 2001), in which perlite is used as a culture substrate, by changing the timing of cryoprotectant addition to preculturing cryotubes from before to after preculture, yielding the modified perlite protocol (MPP) (Sato *et al*., 2012, 2019). Furthermore, we developed the new vermiculite protocol (VP), in which vermiculite instead of perlite is used as a culture substrate, for application to some highly cryosensitive strains of ectomycorrhizal basidiomycete cultures in MPP (Sato *et al*., 2020).

Although the VP is superior to the HPP for short-term (≤1 year) cryopreservation, the HPP is superior to the VP in that the preparation of preculture tubes is easier. Perlite breathes more than vermiculite, and whether this property affects the long-term

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Accepted: April 14, 2021

cryopreservation of specific fungal species is worth examining. Because the resistance to cryoinjury differs at the species and strain levels, examination of the efficacy of long-term cryopreservation using the HPP would be useful. HPP is well known as a good long-term cryopreservation method for saprophytic basidiomycetes (Homolka, 2014), however little information is available on the effectiveness of this method for the long-term cryopreservation of ectomycorrhizal fungi. The development of an effective long-term cryopreservation method is required for safe, stable maintenance of microbial strains, including ectomycorrhizal basidiomycetes; the development of methodological options for laboratories with different types of equipment is also important.

Here, we evaluated the HPP by examining the revival rates of 105 cryosensitive ectomycorrhizal basidiomycete strains after 5 years storage at −80°C. The revival rate and hyphal growth of the revived cultures of 25 selected cryosensitive strains were compared with those of frozen cultures stored at about −170°C for 6 years.

MATERIALS AND METHODS

Strains and culture condition

This work was conducted with 105 basidiomycete strains of 33 species maintained at the National Institute of Technology and Evaluation's Biological Resource Center (NBRC; Table 1). The preculturing, freezing, and revival conditions of 102 strains were the same as in our previous study (Sato *et al*., 2019), and the conditions for the remaining three strains (NBRC 32188 and 32189 *Lyophyllum shimeji* and NBRC 108266 *Tricholoma bakamatsutake*) were modified slightly (Table 1). Twenty-five strains that showed poor or fluctuating viability after 5 years storage at −80°C were selected to compare the survival of frozen cultures after storage at different temperatures (Table 2). Of the 25 strains, 16 were used to compare hyphal growth after revival from frozen cultures.

HPP application

Cultures were frozen using the HPP and thawed as described in Sato *et al*. (2019). Briefly, 0.3 g perlite grains (King Pearl; Iwatani Agri Green Tokyo, Japan) were placed in a cryotube, soaked in 0.8 m*l* liquid medium containing 5% (v/v) glycerol as a cryoprotectant, and autoclaved. A 5-mm-diameter agar plug inoculum was placed on the perlite grains in the cryotube. The inoculated cryotubes were incubated following the preculture conditions given in Table 1. The precultured cryotubes were placed in a freezing container (Mr. Frosty, catalog no. 5100-0001; Thermo Fisher Scientific, Waltham, MA, USA), which was placed in a −80°C freezer, and stored at a −80°C freezer or −170°C in the liquid nitrogen tank in vapor phase until the recovery tests. After storage for 5 years in the −80°C freezer or 6 years in the −170°C in the liquid nitrogen tank in vapor phase, the frozen tubes were thawed rapidly at 30°C in a water bath. The thawed samples were scooped out of the cryotubes with a sterilized spatula and transferred to culture tubes containing 5 m*l* liquid culture medium, which had the same components as the preculture medium except agar and incubated for up to 6 weeks.

Viability test for HPP-preserved samples

Viability was evaluated by counting the number of the 10 revived tubes of each strain showing positive mycelial growth after 6 weeks incubation. We defined a revival rate of 8 or mor out of 10 as "good revival" and 7 or less out of 10 as "poor revival". For the 16 selected strains, the colony diameters of the revived cultures on agar plates after 5 years storage at −80°C and 6 years storage in the vapor-phase liquid nitrogen tank were measured using a ruler at the timepoints shown in Table 3. Differences in the diameters of the revived cultures between the two conditions were compared using Student's *t*-test (two-way), with a significance level of $p<0.05$. Microsoft Excel 365 (Microsoft, Redmond, WA, USA) was used for the calculations.

RESULTS AND DISCUSSION

Long-term preservation of basidiomycete stored at −80°C for 5 years by HPP

The effectiveness of the HPP for the cryopreservation of various ectomycorrhizal basidiomycetes was examined based on the survival rates of 105 strains belonging 33 species after 5 years storage at −80°C. The revived tube numbers (Table 1) correspond to the results of our previous experiments with the same lot (Sato *et al*., 2019). Of the 105 strains tested, 86 (≥80%) showed good revival after 5 years storage. At the species level, the recovery rates indicated good revival for all strains of *Hebeloma spoliatum*, *Lactarius chrysorrheus*, *Lyophyllum decastes*, *Ly. gibberosum*, *Ly. tylicolor*, and *Ly. ulmarium*. However,

Table 1 Numbers of revived tubes of 105 of ectomycorrhizal basidiomycetes strains after 5 years storage at −80°C using the HPP

NBRC No.	Species	Culture conditions			Number of revived tubes in each storage periods[c]		
		Culture medium No.[a]	Pre-culture period (weeks)	Culture period in HPP (weeks)[b]	2 weeks[d]	1 year[d]	5 years
8262	*Amanita aspera* (Persoon) Persoon	7	5	4	10	10	10
8261	*Amanita citrina* Persoon	26	5	6	0	6	0
30376	*A. citrina*	7	5	6	10	10	10
8264	*Amanita muscaria* (Linnaeus) Lamarch	7	4	4	8	10	10
32772	*Amanita pseudoporphyria* Hongo	7	4	4	0	8	10
8263	*Amanita spissa* (Fries) P. Kummer	7	5	5	0	0	0
30329	*Hebeloma spoliatum* (Fries) Gillet	7	3	3	10	10	10
30351	*H. spoliatum*	7	3	3	10	10	10
32944	*H. spoliatum*	7	3	3	10	10	10
33200	*H. spoliatum*	7	5	3	10	10	10
9353	*Kobayasia nipponica* (Kobayasi) S. Imai & A. Kawamura	7	4	5	10	10	10
32775	*Lactarius chrysorrheus* Fries	7	6	6	10	10	10
32791	*La. chrysorrheus*	7	5	3	10	10	10
32794	*Lactarius hatsudake* Nobuj. Tanaka	7	5	5	2	0	0
33155	*La. hatsudake*	7	5	5	7	4	2
7635	*Lyophyllum carbonarium* (Velenovsky) M.M. Moser	1	5	5	10	10	10
30976	*Lyophyllum anthracophilum* (Lasch) M. Lange & Silvertsen	7	5	6	9	10	10
30161	*Lyophyllum decastes* (Fries) Singer	1	4	4	10	10	10
30260	*Ly. decastes*	1	4	4	10	10	8
31167	*Ly. decastes*	1	4	5	10	10	4
32184	*Ly. decastes*	1	4	3	10	10	10
32185	*Ly. decastes*	1	4	4	10	10	10
32186	*Ly. decastes*	1	4	6	10	10	10
33134	*Ly. decastes*	1	4	5	10	10	10
32792	*Lyophyllum fumosum* (Persoon) P.D. Orton	1	5	5	10	10	10
30331	*Lyophyllum gibberosum* (J. Schaeffer) M. Lange	7	4	4	10	10	10
30977	*Ly. gibberosum*	7	3	3	10	10	10
33135	*Lyophyllum semitale* (Fries) Kühner	7	4	5	10	10	10
32187	*Lyophyllum shimeji* (Kawamura) Hongo	26	4	4	10	10	10
32188	*Ly. shimeji*	26	4	4	n.d.	10	4
32189	*Ly. shimeji*	26	4	4	n.d.	2	0
32779	*Ly. shimeji*	26	4	4	5	9	2
32781	*Ly. shimeji*	26	4	4	5	9	10
32809	*Ly. shimeji*	26	4	4	10	10	9
33133	*Ly. shimeji*	26	4	4	2	9	2
101640	*Ly. shimeji*	26	2	4	8	10	9
30779	*Lyophyllum sykosporum* Hongo & Clémençon	7	4	6	10	9	2
30978	*Ly. sykosporum*	1	4	4	10	10	10
32783	*Ly. sykosporum*	1	4	6	10	3	2
30332	*Lyophyllum tylicolor* (Fries) M. Lange & Silvertsen	1	4	4	10	10	10
30486	*Ly. tylicolor*	1	5	4	10	10	10
30487	*Ly. tylicolor*	1	5	4	10	10	10
9637	*Lyophyllum ulmarium* (Bulliard) Kühner	1	4	4	10	10	10
30525	*Ly. ulmarium*	1	4	4	10	10	10
30775	*Ly. ulmarium*	1	4	4	10	10	10

Table 1 Continued

NBRC No.	Species	Culture conditions			Number of revived tubes in each storage periods[c]		
		Culture medium No.[a]	Pre-culture period (weeks)	Culture period in HPP (weeks)[b]	2 weeks[d]	1 year[d]	5 years
100329	*Phallus impudicus* Linnaeus	1	5	5	10	10	8
100327	*Rhizopogon luteolus* Fries	26	4	4	8	2	6
32812	*Rhizopogon rubescens* (Tulasne & C. Tulasne) Tulasne & C. Tulasne	7	3	4	10	10	10
32813	*R. rubescens*	7	3	4	9	10	7
33151	*R. rubescens*	7	3	4	10	10	10
33152	*R. rubescens*	7	3	4	10	10	10
33153	*R. rubescens*	7	3	4	10	10	10
101639	*R. rubescens*	7	3	4	10	10	8
32815	*Sarcodon asparatus* (Berkeley) S. Ito	26	6	6	0	4	3
104563	*Suillus tomentosus* Singer	7	4	4	10	10	9
108265	*Tricholoma bakamatsutake* Hongo	26	5	5	10	10	6
108266	*T. bakamatsutake*	26	5	5	n.d.	9	8
33142	*Tricholoma flavovirens* (Persoon) S. Lundell	7	5	5	9	10	6
33143	*T. flavovirens*	26	5	5	3	0	1
6940	*Tricholoma fulvocastaneum* Hongo	7	5	5	10	10	10
6941	*T. fulvocastaneum*	7	5	5	10	10	10
6942	*T. fulvocastaneum*	7	5	5	10	10	10
6943	*T. fulvocastaneum*	7	5	5	8	10	10
6944	*T. fulvocastaneum*	7	5	5	7	7	9
6945	*T. fulvocastaneum*	7	5	5	9	10	10
6946	*T. fulvocastaneum*	7	5	5	8	8	10
6947	*T. fulvocastaneum*	7	5	5	6	5	10
6949	*T. fulvocastaneum*	7	5	5	10	6	10
108269	*T. fulvocastaneum*	26	5	5	10	10	10
108271	*T. fulvocastaneum*	26	5	5	10	10	10
31860	*Tricholoma giganteum* Massee	7	5	4	10	10	10
32819	*Tricholoma japonicum* Kawamura	7	5	5	10	10	10
32820	*T. japonicum*	7	5	5	9	10	10
6916	*Tricholoma matsutake* (S. Ito & S. Imai) Singer	26	5	5	10	10	10
6917	*T. matsutake*	26	5	4	10	10	10
6918	*T. matsutake*	7	5	4	5	10	10
6920	*T. matsutake*	7	5	6	5	7	10
6922	*T. matsutake*	26	6	6	8	8	9
6924	*T. matsutake*	7	6	4	10	8	8
6925	*T. matsutake*	26	6	4	10	10	8
6926	*T. matsutake*	7	6	4	4	8	10
6928	*T. matsutake*	26	6	4	8	10	10
6929	*T. matsutake*	7	6	4	10	10	10
6930	*T. matsutake*	26	6	4	10	10	8
6931	*T. matsutake*	7	6	4	10	10	10
6932	*T. matsutake*	7	6	4	5	9	10
6933	*T. matsutake*	7	6	4	10	10	10
6934	*T. matsutake*	7	6	4	10	10	10
6935	*T. matsutake*	7	6	4	7	10	10
30604	*T. matsutake*	7	6	4	10	10	10
30605	*T. matsutake*	7	6	4	8	10	10
30606	*T. matsutake*	7	6	4	10	10	10

Table 1 Continued

NBRC No.	Species	Culture conditions			Number of revived tubes in each storage periods[c]		
		Culture medium No.[a]	Pre-culture period (weeks)	Culture period in HPP (weeks)[b]	2 weeks[d]	1 year[d]	5 years
33136	*T. matsutake*	7	6	4	0	4	7
33137	*T. matsutake*	7	6	4	9	10	10
108254	*T. matsutake*	26	6	4	10	10	10
108257	*T. matsutake*	26	6	5	10	10	10
108264	*T. matsutake*	26	6	5	10	10	9
33144	*Tricholoma portentosum* (Fries) Quélet	7	4	5	8	4	3
6936	*Tricholoma robustum* (Albertini & Schweinitz) Ricken	7	4	5	10	9	10
8332	*T. robustum*	7	4	5	10	10	10
32824	*T. robustum*	7	4	5	10	10	10
32808	*Tricholoma ustale* (Fries) P. Kummer	7	4	5	10	10	10
32825	*T. ustale*	7	4	3	10	10	8
33140	*T. ustale*	7	4	5	7	10	10
33141	*T. ustale*	7	4	5	10	9	9

Culture temperature in all experiments was 25°C. [a]Culture medium No. 1: PDA, No. 7: Matsutake medium, No. 26: Mycorrhiza medium. [b]Culture period for incubating mycelia on perlite in cryotube. [c]Ten tubes were tested in each experiment. [d]Data of our previous work (Sato *et al*., 2019). n.d.: no data

19 strains of 13 species in 6 genera (≤70%) revived poorly. As expected, the following six strains that had shown low viability after 2 weeks and 1 year storage (Sato *et al*., 2019) had poor revival after 5 years storage: NBRC 8261 *Amanita citrina*, NBRC 8263 *A. spissa*, NBRC 32794 and 33155 *La. hatsudake*, NBRC 32815 *Sarcodon asparatus*, and NBRC 33143 *Tricholoma flavovirens*. Eight strains with unstable or fluctuating viability during short-term storage (Sato *et al*., 2019; NBRC 32189, 32779, and 33133 *Ly. shimeji*; NBRC 32783 *Ly. sykosporum*; NBRC 100327 *Rhizopogon, luteolus*; NBRC 32813 *R. rubescens*; NBRC 33136 *T. matsutake*; and NBRC 33144 *T. portentosum*) also had poor revival rates after 5 years storage. Furthermore, the following five strains that had shown good short-term storage (Sato *et al*., 2019) had poor revival scores after 5 years storage: NBRC 31167 *Ly. decastes*, NBRC 32188 *Ly. shimeji*, NBRC 30779 *Ly. sykosporum*, NBRC 108265 *T. bakamatsutake*, and NBRC 33142 *T. flavovirens* (Table 1). Of the 105 strains tested, 86 strains achieved good viability after cryopreservation at −80°C, indicating that most of the strains tested can be cryopreserved in an ultra-low-temperature freezer. By contrast, 19 strains had poor revival rates after cryopreservation using the HPP. These strains included ones that had shown poor, unstable, and even stable viability after ≤1 year storage. These results, especially for the latter cases, suggest that the frozen cultures are damaged during storage in a ultra-low-temperature freezer for 5 years.

The effects of the storage temperature on viability and hyphal growth after 5 or 6 years of preservation

Table 2 shows the recovery rates of the 25 selected cryosensitive strains after 5 years storage in an ultra-low-temperature freezer and 6 years storage in the vapor phase of liquid nitrogen. Of the 25 strains, 18 showed no difference or only slight differences in viability, i.e., the numbers of revived tubes were within two of each other. However, the other seven strains (NBRC 32188 and 32189 *Ly. shimeji*, NBRC 30779 and 32783 *Ly. sykosporum*, NBRC 108265 *T. bakamatsutake*, NBRC 33142 *T. flavovirens*, and NBRC 33144 *T. portentosum*) had superior revival rates after storage in vapor-phase liquid nitrogen, with larger differences in the numbers of revived tubes (average 5.7, range 4–10). Comparison of the cryopreservation efficiency according to storage temperature (−80°C and −170°C) revealed significantly larger diameters of the colonies of seven revived strains after storage in vapor-phase liquid nitrogen ($p<0.05$, Table 3), but no significant difference for the

Table 2 Numbers of revived tubes of 25 cryosensitive or fluctuating strains after 6 years storage at −170°C compared with those after 5 years strorage at −80°C both using the HPP

NBRC No.	Species	Number of revived tubes[a]			
		−80°C			−170°C
		2 weeks[b]	1 year[b]	5 years	6 years
8261	*Amanita citrina*	0	6	0	0
32772	*Amanita pseudoporphyria*	0	8	10	10
8263	*Amanita spissa*	0	0	0	0
32794	*Lactarius hatsudake*	2	0	0	2
33155	*La. hatsudake*	7	4	2	3
32188	*Lyophyllum shimeji*	n.d.	10	4	10
32189	*Ly. shimeji*	n.d.	2	0	10
32779	*Ly. shimeji*	5	9	2	0
32781	*Ly. shimeji*	5	9	10	8
32809	*Ly. shimeji*	10	10	9	10
33133	*Ly. shimeji*	2	9	2	0
101640	*Ly. shimeji*	8	10	9	9
30779	*Lyophyllum sykosporum*	10	9	2	8
32783	*Ly. sykosporum*	10	3	2	6
100327	*Rhizopogon luteolus*	8	2	6	6
32815	*Sarcodon asparatus*	0	4	3	2
108265	*Tricholoma bakamatsutake*	10	10	6	10
108266	*T. bakamatsutake*	n.d.	9	8	9
33142	*Tricholoma flavovirens*	9	10	6	10
33143	*T. flavovirens*	3	0	1	0
6944	*Tricholoma fulvocastaneum*	7	7	9	10
6946	*T. fulvocastaneum*	8	8	10	10
6947	*T. fulvocastaneum*	6	5	10	10
6949	*T. fulvocastaneum*	10	6	10	10
33144	*Tricholoma portentosum*	8	4	3	9

[a]Ten tubes were tested in each experiment. [b]Data of our previous work (Sato *et al*., 2019). n.d.: no data

other nine strains. Seven strains had better revival rates after storage in vapor-phase liquid nitrogen. The cryopreservation storage temperature is one of the most important factors for better cryopreservation of bioresources, including basidiomycete cultures. The present study proved that storage in the lower temperature (−170°C) resulted in the better recovery even after the longer storage period than −80°C storage. It is generally recommended that the storage temperature is below the glass transition temperature of water (−130°C) to avoid biochemical reactions and recrystallization processes (Mazur, 1963; Mazur *et al*., 1972). The seven strains might have suffered cryoinjury for 5 years storage at −80°C.

In conclusion, cryopreservation in an ultra-low-temperature freezer using the HPP is useful for the maintenance of many mycorrhizal basidiomycete cultures for at least 5 years. Cryopreservation in vapor-phase liquid nitrogen is more effective for long-term cryopreservation of cryosensitive fungal cultures. This information on long-term preservation at two different storage temperatures might aid the selection of cryopreservation methods. Because researchers studying long-term preservation must overcome difficulties in planning, manpower, equipment, and funding (Linde *et al*., 2018), information on the cryopreservation of basidiomycete cultures for 5 years is limited (Ito & Yokoyama, 1987; Ito & Nakagiri, 1996; Mata *et al*., 2004; Homolka *et al*., 2010). Our study contributes useful information for the stable maintenance and preservation of various basidiomycete cultures for long periods.

ACKNOWLEDGMENTS

We thank Ryutaro Inoue and Tomomi Sasaki for providing technical support, and Yasuyoshi Nakagawa (Director, IPOD-NBRC) for supporting our investigation. This research was supported by the Japan Patent Office.

Table 3 Colony diameters of 16 cryosensitive strains revived after long-term preservation at −80°C and −170°C using the HPP

NBRC No.	Species	Culture period after thawing (weeks)	Colony diameter after thawing (mm: mean±s.d.)		Student's *t*-test p-value −80°C vs. −170°C
			−80°C	−170°C	
32772	*Amanita pseudoporphyria*	4	25.3±3.6	34.4±2.8	5.867E-06*
32188	*Lyophyllum shimeji*	4	24.5±14	49.2±14.5	0.036*
32781	*Ly. shimeji*	2	20.9±10.4	25.8±9.4	0.466
32809	*Ly. shimeji*	4	37.4±3.7	53.4±10.5	0.005E-01*
101640	*Ly. shimeji*	2	38.9±8.5	57.3±5.3	0.005E-02*
30779	*Lyophyllum sykosporum*	2	17.6±13.8	31.3±0.8	0.158
32783	*Ly. sykosporum*	4	32.1±27.7	31.0±3.2	0.948
100327	*Rhizopogon luteolus*	4	13.8±3.4	15.3±8.5	0.723
108265	*Tricholoma bakamatsutake*	5	18.4±2.8	37.0±9.3	0.022*
108266	*T. bakamatsutake*	4	25.3±8.8	30.6±9.5	0.221
33142	*Tricholoma flavovirens*	6	24.1±5.5	42.9±6.6	0.002E-01*
6944	*Tricholoma fulvocastaneum*	6	36.7±2.5	37.5±3.1	0.608
6946	*T. fulvocastaneum*	6	36.3±5.5	37.0±2.6	0.737
6947	*T. fulvocastaneum*	4	23.4±3	25.4±2.6	0.127
6949	*T. fulvocastaneum*	5	31.3±3.8	32.2±5.6	0.692
33144	*Tricholoma portentosum*	6	6.3±4.4	17.6±5.8	0.031*

*Significantly different value in Student's t-test $p<0.05$

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パーライト法による超低温槽と液体窒素気相での保管による担子菌株の長期凍結保存

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Homolka のパーライト法による超低温槽での担子菌培養株 105 株の長期凍結保存性の評価を行った．復元した 105 株のうち 86 株は 5 年間の凍結保存後も良好な生残性を示し，一方 19 株について不良な生残性であった．次に生残性が不良か不安定な 25 菌株を選抜し，保管温度の違いが生残性に与える影響を調べたところ，液体窒素気相（−170℃）で凍結保存した 25 株のうち 7 株において，超低温槽（−80℃）で凍結保存した場合よりも明らかに良好な生残性を示した．さらに，これら 25 株のうちで復元後のコロニー直径の大きさの比較が可能な 16 株においてコロニー直径の統計学的比較を行ったところ，7 株について液体窒素気相保存したもののコロニー直径が −80℃ で凍結保存した場合よりも有意に大きかった．これらの結果から，Homolka のパーライト法を用いた超低温槽での凍結保存法は幅広い種類の菌根性担子菌培養株の長期凍結保存に有効であることを示すとともに，液体窒素気相での凍結保存はさらに安定した保存に有効であることを示した．